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(54) **EPIMERASE**

(57) An acylglucosamine 2-epimerase, a derivative thereof, a DNA coding for the enzyme, a recombinant vector containing the enzyme, a transformant containing the vector integrated therein, a process for producing the enzyme, and a process for producing N-acetylmannosamine and N-acetylneuraminic acid with the use of a resin-binding protein.

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DescriptionField of the Invention

5 The invention relates to acylglucosamine 2-epimerase and derivatives thereof, a DNA molecule encoding the enzyme, a recombinant vector integrated therein to the DNA molecule, a transformant containing the vector and a method for producing the epimerase.

10 The invention relates to a novel polypeptide, acylglucosamine 2-epimerase and derivatives thereof having renin binding activities, a DNA molecule encoding the enzyme, a recombinant vector integrating therein to the DNA molecule, a transformant containing the vector, a method for producing the epimerase, an antihypertensive agent containing the enzyme or derivative thereof as an essential component, an epimerization agent and methods for producing N-acetylmannosamine and N-acetylneuraminic acid.

Background Art

15 In recent years, N-acetylneuraminic acid is noted as raw materials of drugs. It is known that said N-acetylneuraminic acid may be enzymatically synthesized from N-acetylmannosamine and pyruvic acid using N-acetylneuraminic acid lyase. However, because of expensiveness and difficulty of large-scale preparation of N-acetylmannosamine, a method for preparing N-acetylneuraminic acid by reacting inexpensive N-acetylglucosamine and pyruvic acid in the presence of
20 N-acetylneuraminic acid lyase is proposed (Udo Kragl et al., Angewandte Chemi-International Edition in English, 30, 827-828 (1991)). This method utilizes that acylglucosamine 2-epimerase epimerizes N-acetylglucosamine to N-acetylmannosamine. However, acylglucosamine 2-epimerase employed in this method exists only in a trace amount in animal tissues and techniques of large-scale production thereof has not been developed. Accordingly, above-mentioned method may not be employed practically.

25 On the other hand, Teshima et al. (Clinical Chemistry, 34, 2291-2294 (1988)) disclose that acylglucosamine 2-epimerase is useful for determination of N-acetylhexosamine.

As shown above, acylglucosamine 2-epimerase is a very important enzyme and establishment of an efficient method for production thereof is earnestly desired.

30 It is known that acylglucosamine 2-epimerase exists in animal tissues. For example, Asis Datta (Methods in Enzymology, 41, 407-412 (1975)) reported that acylglucosamine 2-epimerase existed in porcine kidney. It also widely exists in kidney, liver, mucosal cell, submandibular gland, intestinal mucosa, colon, salivary gland, etc.

Purification of acylglucosamine 2-epimerase from animal tissues is, however, very difficult, and only crude acylglucosamine 2-epimerase is obtained up to the present. For example, Ghosh et al (Methods in Enzymology, 8, 191-195 (1966)) and Asis Datta (Methods in Enzymology, 41, 407-412 (1975)) tried to isolate and purify acylglucosamine 2-epimerase. However, degree of purity is low according to the report of Ghosh. According to Asis Datta, specific activity
35 thereof is about as low as 6 unit/mg protein.

These reports demonstrate that purification of enzyme from crude extract of porcine kidney cortex prepared by homogenizer followed by a combination of conventional purification means, such as protamine concentration, bentonite treatment, DEAE-cellulose column chromatography, adsorption on calcium phosphate gel, etc. is difficult.

40 The inventors further conduct gel filtration, hydroxyapatite, hydrophobic gel and like a variety of chromatographies and chromatofocusing in addition to said purification means, which do not lead to recovering said enzyme in a purified form due to dilution of enzymatic activities and loss of enzymatic activities caused by inactivation of the enzyme. A trace amount of existence of the enzyme in kidney is one of reasons for difficulties of purification thereof.

45 Recently, preparation of heterologous proteins using microorganisms becomes relatively easy with the progress of gene recombination techniques. However, because of necessity of isolation of protein for utilizing said means, materials to specify said enzyme, such as DNA probes and antibodies may not be prepared with respect to acylglucosamine 2-epimerase which is obtained only in a crude form. In that case, a conventional alternative method comprises electrophoresis of a partially purified enzyme on polyacrylamide gel, blotting the enzyme on polyvinylidenedifluoride (PVDF) membrane to analyze amino acid sequence thereof, synthesizing DNA probes based on said amino acid sequence to
50 detect a desired gene. However, with respect to this enzyme, the amino acid sequence may not be determined by said method, because N-terminal of acylglucosamine 2-epimerase is blocked by an unknown residue.

As shown above, any method generally employed as gene recombination techniques may not be applicable to acylglucosamine 2-epimerase. A way of producing this enzyme by gene recombination techniques has been closed.

55 It is an object of the invention to provide a method for producing acylglucosamine 2-epimerase in large quantities at low cost.

In addition, it is another object of the invention to provide acylglucosamine 2-epimerase.

Further, it is another object of the invention to provide DNA molecules coding for acylglucosamine 2-epimerase.

Furthermore, it is another object of the invention to provide recombinant vectors into which a DNA molecule coding for acylglucosamine 2-epimerase is integrated.

Furthermore, it is another object of the invention to provide transformants, wherein recombinant vectors into which a DNA molecule coding for acylglucosamine 2-epimerase is integrated is introduced.

Furthermore, it is another object of the invention to provide antihypertensive agents.

Furthermore, it is another object of the invention to provide epimerizing agents converting N-acetylglucosamine to N-acetylmannosamine.

Furthermore, it is another object of the invention to provide methods for producing N-acetylmannosamine.

Furthermore, it is another object of the invention to provide methods for producing N-acetylneuraminic acid.

Furthermore, it is another object of the invention to provide novel polypeptides.

Brief Description of the Drawings

Fig. 1 is a scheme showing a nucleotide sequence and an amino acid sequence of acylglucosamine 2-epimerase.

Fig. 2 is a scheme showing a restriction map of plasmid pEP11.

In Fig. 2, □ demonstrates pBluescript which is a vector DNA. ■ demonstrates a DNA inserted. AGE demonstrates a region of gene coding for acylglucosamine 2-epimerase. Plac demonstrates lac promoter.

Fig. 3 is a chromatogram analyzed by HPLC of a reaction mixture modified by PMP. The reaction mixture was prepared by reacting extracts either from cells transformed by recombinant plasmids or cells without transformation in a reaction medium containing N-acetylmannosamine as a substrate. In Fig. 3, GlcNAc demonstrates N-acetylglucosamine modified by PMP; ManNAc demonstrates N-acetylmannosamine modified by PMP.

Fig. 4 is a diagram showing immunological staining after SDS-electrophoresis and western blotting of purified acylglucosamine 2-epimerase derived from porcine kidney and cell extracts of *E. coli*.

In Fig. 4, lane 1 corresponds to purified acylglucosamine 2-epimerase derived from porcine kidney. Lane 2 corresponds to extract of *Escherichia coli*. Lane 3 corresponds to extract of *Escherichia coli* transformed by pEP11. Lane 4 corresponds to extract of *Escherichia coli* transformed by pEP114.

Fig. 5 shows elution pattern of the partially purified enzyme after passing through a hydroxyapatite column. Solid line demonstrates elution of protein determined by ultraviolet absorption (280nm); Broken line demonstrates activities of acylglucosamine 2-epimerase. Collected fractions are shown by arrows.

Disclosure of the Invention

The inventors conducted extensive research in considering the problems of said prior art, and purified acylglucosamine 2-epimerase in a sufficient degree to allow production of antibody so as to conduct cloning and production of acylglucosamine 2-epimerase using said antibody according to gene engineering techniques. In addition, examination of a variety of biological activities of acylglucosamine 2-epimerase thus obtained becomes clear that acylglucosamine 2-epimerase surprisingly has renin binding activities. The invention has been accomplished based on the findings.

Thus, the invention provides an acylglucosamine 2-epimerase and derivatives thereof, DNA molecules encoding the enzyme, recombinant vectors integrated therein to the DNA molecule, transformants containing the vector and methods for producing the epimerase, antihypertensive agents containing the enzyme or derivatives thereof as an essential component, epimerizing agents and methods for producing N-acetylmannosamine and N-acetylneuraminic acid according to items 1-22 shown below.

Item 1. A substantially pure acylglucosamine 2-epimerase.

Item 2. An acylglucosamine 2-epimerase or a derivative thereof having said enzymatic activities comprising an amino acid sequence of the following formula (A) or a amino acid sequence of formula (A) a part of which is replaced or deleted (provided that said amino acid sequence, a part of which is replaced or deleted, has acylglucosamine 2-epimerase

activities.).

(A)

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Met Glu Lys Glu Arg Glu Thr Leu Gln Ala Trp Lys
Glu Arg Val Gly Gln Glu Leu Asp Arg Val Met Ala
Phe Trp Leu Glu His Ser His Asp Arg Glu His Gly
Gly Phe Phe Thr Cys Leu Gly Arg Asp Gly Arg Val
Tyr Asp Asp Leu Lys Tyr Val Trp Leu Gln Gly Arg
Gln Val Trp Met Tyr Cys Arg Leu Tyr Arg Lys Leu
Glu Arg Phe His Arg Pro Glu Leu Leu Asp Ala Ala
Lys Ala Gly Gly Glu Phe Leu Leu Arg His Ala Arg
Val Ala Pro Pro Glu Lys Lys Cys Ala Phe Val Leu
Thr Arg Asp Gly Arg Pro Val Lys Val Gln Arg Ser
Ile Phe Ser Glu Cys Phe Tyr Thr Met Ala Met Asn
Glu Leu Trp Arg Val Thr Ala Glu Ala Arg Tyr Gln
Ser Glu Ala Val Asp Met Met Asp Gln Ile Val His
Trp Val Arg Glu Asp Pro Ser Gly Leu Gly Arg Pro
Gln Leu Pro Gly Ala Val Ala Ser Glu Ser Met Ala
Val Pro Met Met Leu Leu Cys Leu Val Glu Gln Leu

5 Gly Glu Glu Asp Glu Glu Leu Ala Gly Arg Tyr Ala
 Gln Leu Gly His Trp Cys Ala Arg Arg Ile Leu Gln
 His Val Gln Arg Asp Gly Gln Ala Val Leu Glu Asn
 10 Val Ser Glu Asp Gly Glu Glu Leu Ser Gly Cys Leu
 Gly Arg His Gln Asn Pro Gly His Ala Leu Glu Ala
 15 Gly Trp Phe Leu Leu Arg His Ser Ser Arg Ser Gly
 Asp Ala Lys Leu Arg Ala His Val Ile Asp Thr Phe
 20 Leu Leu Leu Pro Phe Arg Ser Gly Trp Asp Ala Asp
 His Gly Gly Leu Phe Tyr Phe Gln Asp Ala Asp Gly
 25 Leu Cys Pro Thr Gln Leu Glu Trp Ala Met Lys Leu
 Trp Trp Pro His Ser Glu Ala Met Ile Ala Phe Leu
 30 Met Gly Tyr Ser Glu Ser Gly Asp Pro Ala Leu Leu
 Arg Leu Phe Tyr Gln Val Ala Glu Tyr Thr Phe Arg
 35 Gln Phe Arg Asp Pro Glu Tyr Gly Glu Trp Phe Gly
 Tyr Leu Asn Arg Glu Gly Lys Val Ala Leu Thr Ile
 40 Lys Gly Gly Pro Phe Lys Gly Cys Phe His Val Pro
 Arg Cys Leu Ala Met Cys Glu Glu Met Leu Ser Ala
 45 Leu Leu Ser Arg Leu Ala

50 Item 3. A DNA molecule coding for acylglucosamine 2-epimerase.

Item 4. A DNA molecule according to item 3 comprising a nucleotide sequence coding for amino acid sequence of the formula (A) or a nucleotide sequence coding for an amino acid sequence of formula (A) a part of which is replaced or deleted (provided that said amino acid sequence, a part of which is replaced or deleted, codes for polypeptide having acylglucosamine 2-epimerase activities.).

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Item 5. A DNA molecule according to item 4 comprising a nucleotide sequence of the following formula (X):

(X)

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ATG GAG AAG GAG CGC GAA ACT CTG CAG GCC TGG AAG
GAG CGT GTG GGC CAA GAG CTG GAC CGC GTG ATG GCT
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TTC TGG CTG GAG CAC TCC CAC GAT CGG GAG CAC GGG
GGC TTC TTC ACG TGC CTG GGC CGC GAC GGG CGG GTG
15
TAT GAC GAC CTC AAG TAC GTC TGG CTG CAG GGG AGG
CAG GTG TGG ATG TAC TGT CGC CTG TAC CGC AAG CTT
20
GAG CGC TTC CAC CGC CCT GAG CTT CTG GAT GCG GCT
AAA GCA GGG GGC GAA TTT TTG CTG CGC CAT GCC CGA
25
GTG GCA CCT CCT GAA AAG AAG TGT GCC TTT GTG CTG
ACG CGG GAC GGC CGG CCC GTC AAG GTG CAG CGG AGC
30
ATC TTC AGT GAG TGC TTC TAC ACC ATG GCC ATG AAC
GAG CTG TGG AGG GTG ACG GCG GAG GCA CGG TAC CAG
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AGC GAA GCG GTG GAC ATG ATG GAT CAG ATC GTG CAC
TGG GTG CGA GAG GAC CCC TCT GGG CTG GGC CGG CCC
40
CAG CTC CCC GGG GCC GTG GCC TCG GAG TCC ATG GCA
GTG CCC ATG ATG CTG CTG TGC CTG GTG GAG CAG CTC
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5 GGG GAG GAG GAC GAG GAG CTG GCA GGC CGC TAC GCG
 CAG CTG GGG CAC TGG TGC GCT CGG AGG ATC CTG CAG
 CAC GTC CAG AGG GAT GGA CAG GCT GTG CTG GAG AAT
 10 GTG TCG GAA GAT GGC GAG GAA CTT TCT GGC TGC CTG
 GGG AGA CAC CAG AAC CCA GGC CAC GCG CTG GAA GCT
 15 GGC TGG TTC CTG CTC CGC CAC AGC AGC CGG AGC GGT
 GAC GCC AAA CTT CGA GCC CAC GTC ATC GAC ACG TTC
 20 CTG CTA CTG CCT TTC CGC TCC GGA TGG GAC GCT GAT
 CAC GGA GGC CTC TTC TAC TTC CAG GAT GCC GAT GGC
 25 CTC TGC CCC ACC CAG CTG GAG TGG GCC ATG AAG CTC
 TGG TGG CCG CAC AGC GAA GCC ATG ATC GCC TTT CTC
 30 ATG GGC TAC AGT GAG AGC GGG GAC CCT GCC TTA CTG
 CGT CTC TTC TAC CAG GTG GCC GAG TAC ACG TTT CGC
 35 CAG TTT CGT GAT CCC GAG TAC GGG GAA TGG TTT GGC
 TAC CTG AAC CGA GAG GGG AAG GTT GCC CTC ACT ATC
 40 AAG GGG GGT CCC TTT AAA GGC TGC TTC CAC GTG CCG
 CGG TGC CTT GCC ATG TGC GAA GAG ATG CTG AGC GCC
 45 CTG CTG AGC CGC CTC GCC TAG

- 50 Item 6. A recombinant vector into which a DNA molecule coding for acylglucosamine 2-epimerase is integrated.
 Item 7. A recombinant vector according to item 6 comprising a nucleotide sequence coding for the amino acid sequence
 of the formula (A) or a nucleotide sequence coding for an amino acid sequence of formula (A) a part of which is replaced
 or deleted (provided that said amino acid sequence, a part of which is replaced or deleted, codes for polypeptide having
 acylglucosamine 2-epimerase activities.).
 55 Item 8. A recombinant vector according to item 7 comprising a nucleotide sequence coding for the amino acid sequence
 of the formula (X).
 Item 9. A transformant wherein a recombinant vector comprising the DNA molecule of item 3 is introduced into said
 transformant.
 Item 10. A method for producing acylglucosamine 2-epimerase comprising introducing a recombinant vector into which

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a DNA molecule coding for acylglucosamine 2-epimerase is integrated into a cell to form a transformant, culturing said transformant in medium to produce and accumulate acylglucosamine 2-epimerase in culture, and collecting acylglucosamine 2-epimerase from the culture.

Item 11. An acylglucosamine 2-epimerase having renin binding activities.

5 Item 12. An epimerase comprising as an essential component a polypeptide as defined in (1) to (3) below:

(1) a polypeptide comprising as an essential sequence said amino acid sequence represented by the formula (A);

10 (2) a polypeptide represented by the formula (A) in which at least one of positions selected from the group consisting of 10, 13, 21, 23, 27, 33, 45, 47, 51, 71, 72, 76-79, 93, 94, 101, 110, 120, 136, 137, 139, 141, 142, 145, 149, 155, 159, 162, 163, 171, 173, 174, 176, 178, 187, 195, 199-202, 205, 208, 212, 224, 232, 234, 237, 243, 249, 258-261, 263, 266, 267, 269, 270, 272, 275, 282, 287-289, 300, 301, 309, 317, 318, 328, 329, 334, 337, 348, 363, 364, 371, 392, 393, 395, 399, 401 and 402 is replaced or deleted by another amino acid; or

15 (3) a polypeptide formed by adding Pro Ala Pro Ser Pro Ala Pro Thr Pro Ala Cys Arg Gly Ala Glu; Pro Ala Pro Leu Gly Ser Leu Pro Ala Val Pro Thr Arg Glu Gly Ser Lys; or Lys Gly Asn Lys Ser Trp Gln Asp to N-terminal or C-terminal of polypeptide of formula (A); (provided that said polypeptide, in which a part of formula (A) is replaced or deleted, has acylglucosamine 2-epimerase activities.).

Item 13. A polypeptide of any of (1) to (3) below (provided that polypeptides represented by formulae (R-1), (R-2) and (R-3) is excluded.);

20 (1) a polypeptide comprising as an essential sequence said amino acid sequence represented by the formula (A);
(2) a polypeptide represented by the formula (A) in which at least one of positions selected from the group consisting of 10, 13, 21, 23, 27, 33, 45, 47, 51, 71, 72, 76-79, 93, 94, 101, 110, 120, 136, 137, 139, 141, 142, 145, 149, 155, 159, 162, 163, 171, 173, 174, 176, 178, 187, 195, 199-202, 205, 208, 212, 224, 232, 234, 237, 243, 249, 258-261, 263, 266, 267, 269, 270, 272, 275, 282, 287-289, 300, 301, 309, 317, 318, 328, 329, 334, 337, 348, 363, 364, 371, 392, 393, 395, 399, 401 and 402 is replaced or deleted by another amino acid; or

25 (3) a polypeptide formed by adding Pro Ala Pro Ser Pro Ala Pro Thr Pro Ala Cys Arg Gly Ala Glu; Pro Ala Pro Leu Gly Ser Leu Pro Ala Val Pro Thr Arg Glu Gly Ser Lys; or Lys Gly Asn Lys Ser Trp Gln Asp to N-terminal or C-terminal of polypeptide of formula (A);

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(R-1)

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Met Glu Lys Glu Arg Glu Thr Leu Gln Ala Trp Lys
Glu Arg Val Gly Gln Glu Leu Asp Arg Val Val Ala
Phe Trp Met Glu His Ser His Asp Gln Glu His Gly
Gly Phe Phe Thr Cys Leu Gly Arg Glu Gly Arg Val
Tyr Asp Asp Leu Lys Tyr Val Trp Leu Gln Gly Arg
Gln Val Trp Met Tyr Cys Arg Leu Tyr Arg Thr Phe
Glu Arg Phe Arg His Ala Gln Leu Leu Asp Ala Ala
Lys Ala Gly Gly Glu Phe Leu Leu Arg Tyr Ala Arg
Val Ala Pro Pro Gly Lys Lys Cys Ala Phe Val Leu
Thr Arg Asp Gly Arg Pro Val Lys Val Gln Arg Thr
Ile Phe Ser Glu Cys Phe Tyr Thr Met Ala Met Asn
Glu Leu Trp Arg Ala Thr Gly Glu Val Arg Tyr Gln
Thr Glu Ala Val Glu Met Met Asp Gln Ile Val His
Trp Val Gln Glu Asp Ala Ser Gly Leu Gly Arg Pro
Gln Leu Gln Gly Ala Pro Ala Ala Glu Pro Met Ala
Val Pro Met Met Leu Leu Asn Leu Val Glu Gln Leu
Gly Glu Ala Asp Glu Glu Leu Ala Gly Lys Tyr Ala
Glu Leu Gly Asp Trp Cys Ala Arg Arg Ile Leu Gln

His Val Gln Arg Asp Gly Gln Ala Val Leu Glu Asn
Val Ser Glu Gly Gly Lys Glu Leu Pro Gly Cys Leu
Gly Arg Gln Gln Asn Pro Gly His Thr Leu Glu Ala
Gly Trp Phe Leu Leu Arg His Cys Ile Arg Lys Gly
Asp Pro Glu Leu Arg Ala His Val Ile Asp Lys Phe
Leu Leu Leu Pro Phe His Ser Gly Trp Asp Pro Asp
His Gly Gly Leu Phe Tyr Phe Gln Asp Ala Asp Asn
Phe Cys Pro Thr Gln Leu Glu Trp Ala Met Lys Leu
Trp Trp Pro His Ser Glu Ala Met Ile Ala Phe Leu
Met Gly Tyr Ser Asp Ser Gly Asp Pro Val Leu Leu
Arg Leu Phe Tyr Gln Val Ala Glu Tyr Thr Phe Arg
Gln Phe Arg Asp Pro Glu Tyr Gly Glu Trp Phe Gly
Tyr Leu Ser Arg Glu Gly Lys Val Ala Leu Ser Ile
Lys Gly Gly Pro Phe Lys Gly Cys Phe His Val Pro
Arg Cys Leu Ala Met Cys Glu Glu Met Leu Gly Ala
Leu Leu Ser Arg Pro Ala Pro Ala Pro Ser Pro Ala
Pro Thr Pro Ala Cys Arg Gly Ala Glu

(R-2)

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Met Glu Lys Glu Arg Glu Thr Leu Gln Val Trp Lys

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Gln Arg Val Gly Gln Glu Leu Asp Ser Val Ile Ala

Phe Trp Met Glu His Ser His Asp Gln Glu His Gly

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Gly Phe Phe Thr Cys Leu Gly Arg Asp Gly Gln Val

Tyr Asp His Leu Lys Tyr Val Trp Leu Gln Gly Arg

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Gln Val Trp Met Tyr Cys Arg Leu Tyr Arg Thr Phe

Glu Arg Phe Arg Arg Val Glu Leu Leu Asp Ala Ala

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Lys Ala Gly Gly Glu Phe Leu Leu Ser Tyr Ala Arg

Val Ala Pro Pro Gly Lys Lys Cys Ala Phe Val Leu

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Thr Gln Asp Gly Arg Pro Val Lys Val Gln Arg Thr

Ile Phe Ser Glu Cys Phe Tyr Thr Met Ala Met Asn

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Glu Leu Trp Lys Val Thr Gly Glu Met His Tyr Gln

Arg Glu Ala Val Glu Met Met Asp Gln Ile Ile His

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Trp Val Arg Glu Asp Pro Ala Gly Leu Gly Arg Pro

Gln Leu Ser Gly Thr Leu Ala Thr Glu Pro Met Ala

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Val Pro Met Met Leu Leu Asn Leu Val Glu Gln Leu

Gly Glu Glu Asp Glu Glu Met Thr Asp Lys Tyr Ala

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Glu Leu Gly Asp Trp Cys Ala His Arg Ile Leu Gln

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His Val Gln Arg Asp Gly Gln Val Val Leu Glu Asn
 Val Ser Glu Asp Gly Lys Glu Leu Pro Gly Cys Leu
 Gly Arg His Gln Asn Pro Gly His Thr Leu Glu Ala
 Gly Trp Phe Leu Leu Gln Tyr Ala Leu Arg Lys Gly
 Asp Pro Lys Leu Gln Arg His Ile Ile Asp Lys Phe
 Leu Leu Leu Pro Phe His Ser Gly Trp Asp Pro Glu
 His Gly Gly Leu Phe Tyr Phe Gln Asp Ala Asp Asp
 Leu Cys Pro Thr Gln Leu Glu Trp Asn Met Lys Leu
 Trp Trp Pro His Thr Glu Ala Met Ile Ala Phe Leu
 Met Gly Tyr Arg Asp Ser Gly Asp Pro Ala Leu Leu
 Asn Leu Phe Tyr Gln Val Ala Glu Tyr Thr Phe His
 Gln Phe Arg Asp Pro Glu Tyr Gly Glu Trp Phe Gly
 Tyr Leu Asn Gln Glu Gly Lys Val Ala Leu Thr Ile
 Lys Gly Gly Pro Phe Lys Gly Cys Phe His Val Pro
 Arg Cys Leu Ala Met Cys Glu Gln Ile Leu Gly Ala
 Leu Leu Gln Arg Leu Gly Pro Ala Pro Leu Gly Ser
 Leu Pro Ala Val Pro Thr Arg Glu Gly Ser Lys

(R-3)

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Met Glu Lys Glu Arg Glu Thr Leu Gln Ala Trp Lys
Glu Arg Val Gly Gln Glu Leu Asp Arg Val Met Ala
Phe Trp Leu Glu His Ser His Asp Arg Glu His Gly
Gly Phe Phe Thr Cys Leu Gly Arg Asp Gly Arg Val
Tyr Asp Asp Leu Lys Tyr Val Trp Leu Gln Gly Arg
Gln Val Trp Met Tyr Cys Arg Leu Tyr Arg Lys Leu
Glu Arg Phe His Arg Pro Glu Leu Leu Asp Ala Ala
Lys Ala Gly Gly Glu Phe Leu Leu Arg His Ala Arg
Val Ala Pro Pro Glu Lys Lys Cys Ala Phe Val Leu
Thr Arg Asp Gly Arg Pro Val Lys Val Gln Arg Ser
Ile Phe Ser Glu Cys Phe Tyr Thr Met Ala Met Asn
Glu Leu Trp Arg Val Thr Ala Glu Ala Arg Tyr Gln
Ser Glu Ala Val Glu Met Met Asp Gln Ile Val His
Trp Val Arg Glu Asp Pro Ser Gly Leu Gly Arg Pro
Gln Leu Pro Gly Ala Val Ala Ser Glu Ser Met Ala
Val Pro Met Met Leu Leu Cys Leu Val Glu Gln Leu

5 Gly Glu Glu Asp Glu Glu Leu Ala Gly Arg Tyr Ala
 Gln Leu Gly His Trp Cys Ala Arg Arg Ile Leu Gln
 His Val Gln Arg Asp Gly Gln Ala Val Leu Glu Asn
 10 Val Ser Glu Asp Gly Glu Glu Leu Ser Gly Cys Leu
 Gly Arg His Gln Asn Pro Gly His Ala Leu Glu Ala
 15 Gly Trp Phe Leu Leu Arg His Ser Ser Arg Ser Gly
 Asp Ala Lys Leu Arg Ala His Val Ile Asp Thr Phe
 20 Leu Leu Leu Pro Phe Arg Ser Gly Trp Asp Ala Asp
 Tyr Gly Gly Leu Phe Tyr Phe Gln Asp Ala Asp Gly
 25 Leu Cys Pro Thr Gln Leu Glu Trp Ala Met Lys Leu
 Trp Trp Pro His Arg Gln Ala Met Ile Ala Phe Leu
 30 Met Gly Tyr Ser Glu Ser Gly Asp Pro Ala Leu Leu
 Arg Leu Phe Tyr Gln Val Ala Glu Tyr Thr Phe Arg
 35 Gln Phe Arg Asp Pro Glu Tyr Gly Glu Trp Phe Gly
 Tyr Leu Asn Arg Glu Gly Lys Val Ala Leu Thr Ile
 40 Lys Gly Gly Pro Phe Lys Gly Cys Phe His Val Pro
 Arg Cys Leu Ala Met Cys Glu Glu Met Leu Ser Ala
 45 Leu Leu Ser Arg Leu Ala

- 50 Item 14. A DNA molecule coding for polypeptide according to item 13.
 Item 15. A DNA molecule according to item 14 comprising the nucleotide sequence of formula (X).
 Item 16. A recombinant vector into which a DNA molecule coding for acylglucosamine 2-epimerase according to item 14 or 15 is integrated.
 Item 17. A transformant wherein a recombinant vector according to item 16 is introduced into said transformant.
 55 Item 18. A method for producing acylglucosamine 2-epimerase having renin binding activities comprising introducing a recombinant vector into which a DNA molecule coding for acylglucosamine 2-epimerase having renin binding activities is integrated into a cell to form a transformant, culturing said transformant in medium to produce and accumulate acylglucosamine 2-epimerase in culture, and collecting acylglucosamine 2-epimerase from the culture.
 Item 19. An antihypertensive agent comprising as an essential component acylglucosamine 2-epimerase or a derivative

thereof according to item 11 or 12.

Item 20. An epimerizing agent converting N-acetylglucosamine to N-acetylmannosamine comprising as an essential component a polypeptide having renin binding activities.

Item 21. A method for producing N-acetylmannosamine comprising acting a polypeptide having renin binding activities on N-acetylglucosamine.

Item 22. A method for producing N-acetyneuraminic acid comprising acting a polypeptide having renin binding activities and N-acetyneuraminic acid lyase on N-acetylglucosamine and pyruvic acid.

It is known that said proteins (R-1), (R-2) and (R-3) have renin binding activities (H. Inoue et al, J. Biochem., 110, p.493-500 (1991)). However, it is not known that said proteins have acylglucosamine 2-epimerase activities. Thus, "protein having renin binding activities" employed in a method for producing N-acetylmannosamine and a method for producing N-acetyneuraminic acid of the invention includes said proteins (R-1), (R-2) and (R-3).

The invention provides a method for producing N-acetylmannosamine characterized in that at least one of said proteins (R-1), (R-2) and (R-3) is applied to.

Further, the invention provides a method for producing N-acetyneuraminic acid characterized in that at least one of said proteins (R-1), (R-2) and (R-3) and N-acetyneuraminic acid lyase acts on N-acetylglucosamine and pyruvic acid.

"Protein having renin binding activities", an essential component of epimerizing agent of the invention, comprises said proteins (R-1), (R-2) and (R-3).

Therefore, the invention provides an epimerizing agent converting N-acetylglucosamine to N-acetylmannosamine comprising as an essential component at least one selected from the group consisting of said proteins (R-1), (R-2) and (R-3).

The novel polypeptides of the invention are useful as acylglucosamine 2-epimerase.

Since said items 1-10 are enough as long as they have acylglucosamine 2-epimerase activities, irrespective of existence of renin binding activities, items 1-10 are hereinafter referred to as "first invention".

In addition, said items 11-22 relates to proteins having both acylglucosamine 2-epimerase activities and renin binding activities, and hereinafter referred to as "second invention".

Further, "the invention" includes first invention and second invention.

At least some of acylglucosamine 2-epimerases of the invention have acylglucosamine 2-epimerase activities and renin binding activities. Said epimerases include all proteins having acylglucosamine 2-epimerase activities, irrespective of renin binding activities thereof.

According to the invention, cells transformed by recombinant vector into which DNA molecule coding for acylglucosamine 2-epimerase is integrated are not specifically limited. Example of such cells are Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Actinomycetes, Lactic acid bacteria and like bacteria, fungi, yeast and like eucaryotic microorganisms, mouse cells, rat fibroblast, plant cells and like cells as long as cells allow stable retainment and function of plasmids.

For carrying out the invention, skilled artisan may suitably select a method for introducing a plasmid into which a DNA molecule coding for acylglucosamine 2-epimerase is integrated into said cell, according to the type of cells. For example, introduction of plasmid into E. coli may be carried out according to a method of Hanahan (DNA Cloning, Vol.1, p.109-136 (1985)).

The invention first isolates acylglucosamine 2-epimerase in a form substantially free of impurities and discloses a method for production thereof. The invention first discloses that at least a part of said epimerases have renin binding activities. The second invention includes polypeptides having both acylglucosamine 2-epimerase activities and renin binding activities irrespective of amino acid sequence thereof. For example, the invention includes DNA molecules in which several to one hundred and tens of nucleotides at 5' terminal and/or 3' terminal are eliminated by exonuclease, and derivatives of said enzyme encoded by said DNA in which several to tens of amino acids at N-terminal and/or C-terminal are eliminated, as long as said derivatives retain said enzymatic activities. Further, the invention includes polypeptides in which at least one amino acid in the amino acid sequence thereof is deleted or replaced by another amino acid according to known point mutation methods, as long as said polypeptides retain said acylglucosamine 2-epimerase activities.

Positions of polypeptides represented by formula (A) allowing replacement and/or deletion are not specifically limited to, but include 10, 13, 21, 23, 27, 33, 45, 47, 51, 71, 72, 76-79, 93, 94, 101, 110, 120, 136, 137, 139, 141, 142, 145, 149, 155, 159, 162, 163, 171, 173, 174, 176, 178, 187, 195, 199-202, 205, 208, 212, 224, 232, 234, 237, 243, 249, 258-261, 263, 266, 267, 269, 270, 272, 275, 282, 287-289, 300, 301, 309, 317, 318, 328, 329, 334, 337, 348, 363, 364, 371, 392, 393, 395, 399, 401 and 402.

Further, the invention include polypeptides to which several to tens of amino acids at N-terminal and/or C-terminal are added, as long as said polypeptides retain said acylglucosamine 2-epimerase activities.

Donors of nucleic acid molecule coding for acylglucosamine 2-epimerase employed in the invention are not specifically limited to, but include animal tissues having said enzymatic activities, such as porcine and human kidneys, kidney, liver, mucosal cell, submandibular gland, intestinal mucosa, colon, salivary gland, etc. of human or rat. Said nucleic acid molecules are obtained from the animal tissues. The nucleic acid molecules include DNAs and RNAs, preferably RNAs.

RNAs coding for acylglucosamine 2-epimerase may be obtained according to a method of Chomczynski et al (Analytical Biochemistry, 162, 156-159, (1987)). RNAs are also obtained as RNAs with polyadenylate tail (poly A tail) according to a method described in Molecular Cloning Second Edition Vol.1, sections 7.26-7.29. cDNAs coding for acylglucosamine 2-epimerase may be easily obtained by using the RNAs with poly A tail.

Conversion of RNA to cDNA may be carried out, for example, according to Current Protocols in Molecular Biology, Vol.1, 5.5.1-5.5.10 (1990). The conversion may also be carried out by using commercially available cDNA synthesis kit (eg. product of Amersham, Stratagene, etc).

cDNA library may be constructed by inserting cDNA into some vectors, for example, phage vectors and plasmid vectors. Construction of cDNA library may be carried out according to a method described in Molecular Cloning Second Edition Vol.2, sections 8.1-8.86.

Means to select transformant retaining cDNA coding for acylglucosamine 2-epimerase from cDNA library constructed are needed. With respect to said enzyme, purified enzyme with required level in the field of recombination of gene was not obtained as stated above. The inventors obtained acylglucosamine 2-epimerase purified enough to produce antibody according to the following method.

First, acylglucosamine 2-epimerase is partially purified according to the method of Asis Datta (Methods in Enzymology, 41, 407-412 (1975)). In the following purification procedure, fractions with high activities and small amount of proteins, i.e., only fractions whose specific activity is higher than specific activity of sample before elution of column are collected, because fractions having activities spread in the process of purification. Specifically, it is found that said enzyme is purified by applying said partially purified sample to hydroxyapatite column and ion-exchange chromatography in this sequence to collect the eluted fraction with highest enzymatic activities, followed by separation thereof by ion-exchange chromatography repeated two times. The enzyme fraction is further purified by HPLC with reverse-phase column (μ Bondasphere, product of Millipore) to obtain said purified enzyme protein substantially free of impurities. The reverse-phase HPLC is hardly employed for purification of enzymes, since enzymes are easily inactivated during purification. Acylglucosamine 2-epimerase also loses enzymatic activities thereof by said purification, but maintain elicitation ability to produce antibodies. Therefore, antibodies specifically reacted with said enzyme protein are obtained by immunizing rabbit with acylglucosamine 2-epimerase purified by HPLC.

According to said purification steps, for example, 10.5 mg of said purified enzyme protein is obtained from 5.6 kg of porcine kidney cortex.

The DNA molecule coding for acylglucosamine 2-epimerase of the invention may be isolated from constructed cDNAs. The isolation may be carried out by detecting said enzyme with the antibodies using λ gt11, λ ZAP and like vectors as expression vector. The transformant retaining DNA coding for acylglucosamine 2-epimerase produces acylglucosamine 2-epimerase, when exposed by isopropyl- β -D-thiogalactopyranoside (IPTG). The transformant retaining DNA coding for said enzyme may be selected and obtained by binding this to antibody, to which anti-rabbit antibody is bound, followed by reacting the complex with 5-bromo-4-chloro-3-indolylphosphate solution and nitroblue tetrazolium solution.

When resulting DNA coding for acylglucosamine 2-epimerase is inserted into plasmid DNA, *E. coli* containing said DNA is cultured in a suitable medium to obtain acylglucosamine 2-epimerase. When λ ZAP is used as a vector, said DNA may be excised cut as a plasmid into vector by simultaneous infection of obtained phage containing said DNA and f1 helper phage. A novel recombinant plasmid containing DNA, for example, represented by formula (X) having 1.2 kbp, coding for acylglucosamine 2-epimerase may be obtained according to the series of operations.

Cells such as *Escherichia coli* are transformed by using plasmid inserted therein a DNA fragment thus obtained coding for acylglucosamine 2-epimerase. In order to produce acylglucosamine 2-epimerase from transformed cells, transformed cells are cultured in the following conditions to obtain mass of cells.

Culture conditions are different depending on types of cells transformed, and are easily determined in accordance with types of cells. For example, *E. coli* may be cultured by conventional solid culture method, but is preferably cultured by liquid culture method. In addition, taking *E. coli* as an example, media employed for culture contain carbon sources, nitrogen sources, inorganic compounds and other nutrient components. Any one of synthetic medium, semisynthetic medium, natural medium and like medium generally employed for culturing bacteria may be used. Examples of carbon sources for said media are glucose, fructose, invert sugar, starch, saccharified starch, sorbitol, glycerol, and like carbohydrate solution, pyruvic acid, malic acid, succinic acid, and like organic acids. Examples of nitrogen sources are ammonium sulfate, ammonium chloride, ammonium nitrate, ammonium phosphate, ammonium hydroxide, ammonium tartrate, ammonium acetate, urea, etc. Substances employed as both carbon and nitrogen sources include peptone, yeast extract, meat extract, corn steep liquor, etc. Examples of inorganic compounds are potassium dihydrogenphosphate, dipotassium hydrogenphosphate, sodium dihydrogenphosphate, disodium hydrogenphosphate, magnesium phosphate, magnesium chloride, potassium chloride, sodium chloride, ferrous sulfate, ferric sulfate, ferric chloride, manganese sulfate, manganese chloride, etc.

Culture time and culture temperature of transformed cells are not specifically limited. Taking *Escherichia coli* as an example of said cells, culture is conducted at generally 20-42 °C, preferably 30-37 °C, for generally 4-48 hours, preferably 8-14 hours. Under these conditions, conventional shake culture or aeration agitation culture is carried out.

When DNA coding for acylglucosamine 2-epimerase of the invention is bound to a suitable vector to transform a suitable host cell, oxygen concentration of inside or outside of transformed host cell is increased leading to efficient production of said enzyme.

The vector employed in the invention comprises the following elements. Specifically, the vector comprises promoter placed in a correct orientation and position to express DNA coding for acylglucosamine 2-epimerase of the invention and translation activating sequence. Any vector comprising these elements may be employed, but preferably are vectors comprising suitable selected marker and multicopy vectors, which include pBluescript, pUC18, pUC19, pKK223-3 and pTrc99A, etc. When these vectors are employed, intracellular concentration of acylglucosamine 2-epimerase may be elevated by adding about 0.01mM-100mM, preferably about 0.1mM-10mM of isopropyl- β -D-thiogalactopyranoside to culture medium. Further, pPL-lambda, heat-induced expression vector, may be employed. When employing this vector, intracellular concentration of acylglucosamine 2-epimerase may be increased by elevating temperature of culture medium to 40-45 °C.

Further, increase of productivity and efficiency of said enzyme may be accomplished by deleting terminal DNA of said DNA coding for acylglucosamine 2-epimerase to increase intracellular concentration of acylglucosamine 2-epimerase. For example, production efficiency of acylglucosamine 2-epimerase may be further increased by degradation of said DNA molecule at 5' and/or 3' terminal with exonuclease, etc., while maintaining acylglucosamine 2-epimerase activities.

In addition, productivity of said enzyme may be increased by site-specific mutation or random mutation to replace or delete said DNA at an internal region. For example, site-specific mutation may be introduced by integrating said DNA molecule into pBluescript, M13 and like vectors capable of becoming single strand to prepare single strand DNA containing said DNA molecule; annealing oligonucleotides containing sequence to be mutated or deleted to said single strand DNA; and conducting primer elongation reaction utilizing said oligonucleotides as a primer in the presence of deoxyribonucleotide triphosphate, ATP, Klenow fragment and T4 ligase.

Acylglucosamine 2-epimerase may be extracted from cultured cells. Extraction may be carried out according to conventional enzyme extraction methods. For example, said enzyme may be collected from supernatant by crushing cells with ultrasonic treatment, a variety of mechanical treatments, enzymatic treatments and like methods, followed by separating insoluble matter by centrifugation. The collected crude enzyme may be purified by suitably combining conventional enzyme purification methods. For example, a great amount of purified acylglucosamine 2-epimerase may be obtained by nucleic acid removal treatment, ammonium sulfate treatment, diatomaceous earth treatment, ion-exchange chromatography, etc.

The *Escherichia coli* strain containing pEP11 of Fig.2 integrated therein to the DNA molecule of Fig.1 is deposited in National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology and accession number: FERM BP-4602 is given.

The inventors conducted extensive research on biological activities of polypeptide of formula (A) derived from porcine kidney obtained in second invention and make clear that said enzyme has not only acylglucosamine 2-epimerase activities but also renin binding activities. "Renin binding activities" mean that said polypeptide binds to renin resulting in inhibition of renin activities. The polypeptide of formula (A) is obtained according to the method for producing acylglucosamine 2-epimerase of the invention. In contrast, renin binding protein from porcine kidney of formula (R-3) is obtained according to a method disclosed by Inoue et al (H. Inoue et al., J. Biochem., 110, p.493-500, (1991)). The two proteins (A) and (R-3) obtained by different purification method are very similar proteins except that they have different amino acids at positions 149, 289, 317 and 318. As shown in the following examples, the polypeptide (A) obtained according to the invention and epimerase from rat kidney have renin binding activities, which indicates that, with respect to at least part of proteins, acylglucosamine 2-epimerase activities and renin binding activities are inseparable. Accordingly, known renin binding proteins (R-1), (R-2) and (R-3) will have epimerase activities. When renin binding proteins have acylglucosamine 2-epimerase activities, said renin binding proteins may be employed as epimerizing agents catalyzing the conversion reaction from N-acetylglucosamine to N-acetylmannosamine.

The protein of formula (A) is different from known proteins (R-1), (R-2) and (R-3) in any one of positions 10, 13, 21, 23, 27, 33, 45, 47, 51, 71, 72, 76-79, 93, 94, 101, 110, 120, 136, 137, 139, 141, 142, 145, 149, 155, 159, 162, 163, 171, 173, 174, 176, 178, 187, 195, 199-202, 205, 208, 212, 224, 232, 234, 237, 243, 249, 258-261, 263, 266, 267, 269, 270, 272, 275, 282, 287-289, 300, 301, 309, 317, 318, 328, 329, 334, 337, 348, 363, 364, 371, 392, 393, 395, 399, 401 and 402. However, these proteins have acylglucosamine 2-epimerase activities and renin binding activities so that these positions are not essential for biological activities and may be replaced or deleted. Further, addition of tens of amino acids to the proteins at N-terminal, C-terminal does not affect expression of activities of the proteins. The inventors, in fact, obtained an epimerase obtained by adding amino acids, Lys Gly Asn Lys Ser Trp Gln Asp, to the polypeptide of formula (A) at N-terminal. It is confirmed that said epimerase has sufficient enzymatic activities.

The invention discloses a method for collecting acylglucosamine 2-epimerase produced intracellularly in large amounts comprising separating a DNA molecule coding for acylglucosamine 2-epimerase from animal tissues, preparing a recombinant plasmid containing said DNA molecule, introducing said plasmid into *E. coli* and like host cell to transform the cell and culturing the transformant.

Renin produces angiotensin I by hydrolyzing angiotensinogen. Angiotensin I is further converted to angiotensin II with angiotensin converting enzyme to express strong hypertensive action by directly constricting smooth muscle of peripheral blood vessel. Angiotensin II also acts on adrenal gland zona glomerulosa to accelerate secretion of aldosterone. As shown above, renin-angiotensin system plays an important role on regulation of blood pressure. Therefore, inhibitors of renin-angiotensin system are developed and widely employed as anti-hypertensive agent. Since acylglucosamine 2-epimerase of the invention binds to renin leading to inhibit activities thereof, acylglucosamine 2-epimerase is useful as an anti-hypertensive agent.

Renin binding proteins have acylglucosamine 2-epimerase activities. Accordingly, renin binding proteins may be employed as epimerizing agent converting N-acetylglucosamine to N-acetylmannosamine. When renin binding proteins act on N-acetylglucosamine, N-acetylmannosamine is obtained. Further, when renin binding proteins and N-acetylneuraminic acid lyase act on N-acetylglucosamine and pyruvic acid, N-acetylneuraminic acid may be obtained by converting N-acetylglucosamine to N-acetylmannosamine with the action of renin binding protein, followed by binding N-acetylmannosamine to pyruvic acid.

According to the invention, the outstanding effects as shown below are exerted.

- (1) Highly purified acylglucosamine 2-epimerase may be obtained in large amounts with low costs.
- (2) Because microorganisms are employed as starting materials, the method of the invention is not limited with respect to supply of raw material unlike conventional method using animal tissues as raw material. Therefore, production thereof may be carried out at a desired place in required amounts in any time.
- (3) Because of high productivity of acylglucosamine 2-epimerase, said enzyme may easily be isolated.
- (4) Because high-purity acylglucosamine 2-epimerase may be obtained with low costs, N-acetylneuraminic acid and N-acetylmannosamine may be produced with low costs.
- (5) Because high-purity acylglucosamine 2-epimerase may be obtained, it may be employed as assay of N-acetylneuraminic acid and N-acetylhexosamine.
- (6) Antihypertensive agents are obtained by using proteins having renin binding activities of the invention.
- (7) Because proteins having renin binding activities also have acylglucosamine 2-epimerase activities, said proteins may be employed as epimerizing agents converting N-acetylglucosamine to N-acetylmannosamine.
- (8) N-acetylneuraminic acid may be efficiently obtained by reacting proteins of the invention with renin binding activities and N-acetylneuraminic acid lyase with N-acetylglucosamine and pyruvic acid optionally under alkaline conditions.

Examples

The invention will be described below in greater detail using examples, but the invention is in no way limited to the examples.

Example 1

Production of acylglucosamine 2-epimerase from porcine kidney

(1) Purification of acylglucosamine 2-epimerase from porcine kidney

Freshly obtained porcine kidney cortex (5.6 kg) supplemented with 12 liter of 3mM phosphate buffer (pH 7.6) was homogenized with homogenizer. After obtaining supernatant (11 liter) by sequential centrifugation (10,000 rpm, 200ml/min), cooled distilled water equal volume to the supernatant was added thereto. The resulting mixture was sufficiently stirred and then sequentially centrifuged (10,000 rpm, 200ml/min) to obtain kidney extract as supernatant. The kidney extract was treated by protamine concentration, bentonite treatment, DEAE-cellulose column chromatography and calcium phosphate gel according to Asis Datta (Methods in Enzymology, 41, 407-412 (1975)) for purification of acylglucosamine 2-epimerase to obtain 387 mg of partially purified enzyme.

Said partially purified enzyme was applied to hydroxyapatite column (inner diameter 26mm x length 95 mm; WAKO PURE CHEMICAL CO., LTD.) equilibrated with 10 mM phosphate buffer (pH 7.6) and eluted with the same buffer. Fractions with said enzymatic activities were eluted in widely spread condition according to the treatments of the invention (see, Fig.5). The 12th to 17th fractions (18ml x 6 = 108 ml) having said enzymatic activities were treated with salting out techniques using ammonium sulfate to collect fractions saturated with 0-80 % by weight of ammonium sulfate. The fractions were dialyzed against 20 mM phosphate buffer (pH 7.6) to give 75.6 mg of dialyzed enzyme. The enzyme was further purified by ion-exchange chromatography in the following conditions to purify the enzyme.

Q-Sepharose (PHARMACIA), one of ion-exchange resins, was filled in a column (inner diameter 26mm x length 95 mm). The column was equilibrated by 20 mM phosphate buffer (pH 7.6; 500 ml). Said partially purified enzyme (75.6 mg) was adsorbed on the column and eluted by linear gradient using phosphate buffer (pH 7.6) containing 100 mM to

300 mM of potassium chloride. A main peak corresponding to a protein eluted with a potassium chloride concentration of about 180 mM (198 ml) was collected, and then concentrated and dialyzed. The dialyzed enzyme (23.0 mg) was applied to Mono Q column (PHARMACIA) to adsorb said enzyme on the column. The adsorbed enzyme was eluted by linear gradient using phosphate buffer (pH 7.6) containing 200 mM to 300 mM of potassium chloride. A peak corresponding to a protein eluted with a potassium chloride concentration of about 220 mM was collected, and then desalted by gel filtration column. An activity of the enzyme (15.9 mg) thus obtained was 21 units/mg protein as a specific activity, which is 3.5 time as high as the activity (purity) of the protein (6 units/mg protein) reported by Asis Datta (Methods in Enzymology, 41, 407-412 (1975)).

Further, the resulting enzyme was purified to remove low-molecular-weight materials and trace amount of impurities by HPLC using reverse phase column. μ Bondasphere 5 μ C4-300 Å (inner diameter 3.9 mm x length 150 mm) (MILLIPORE) was employed as reverse phase column. Said purified enzyme (2 mg) was subjected to the column equilibrated with 0.1 % (V/V) TFA aqueous solution. Said purified enzyme retained in the column was eluted by linear gradient using 0.1 % (V/V) TFA aqueous solution containing 0 to 80 % (V/V) of acetonitrile. A main peak protein determined by ultraviolet absorption (280 nm) was collected and dried in vacuo. Said procedure was repeated 6 times to obtain the enzyme (10.5 mg). The enzyme thus obtained loses biological activities but is substantially free of impurities.

(2) Antibodies specifically bound to acylglucosamine 2-epimerase

Nine weeks old rabbits (JAPANESE WHITE) were immunized with said purified acylglucosamine 2-epimerase (5.2 mg). Blood of said rabbit was gathered partially and wholly to obtain 150 ml of antiserum. IgG was purified from the antiserum using Protein A Sepharose according to the description on page 24 of New Biochemical Experimental Course 12, Ed. Japan Biochemistry organization, Molecular Immunology III, (1992).

Example 2

Cloning of cDNA coding for acylglucosamine 2-epimerase

(1) Production of mRNA from porcine kidney

Kidney cortex was cut out from porcine kidney. RNAs (4.9 mg) were obtained from 2g of kidney cortex according to the method of Chomczynski et al (Analytical Biochemistry, 162, 156, (1987)). RNAs with poly(A) tail (67 μ g) were then obtained by adsorbing said RNA on oligo-dT-cellulose column, followed by elution thereof.

(2) Preparation of cDNA library

A cDNA library was prepared from the RNAs with poly(A) tail (5 μ g) thus obtained using a ZAP-cDNA synthesis kit (STRATAGENE). The library obtained was 4.5×10^{12} of plaque forming unit.

(3) Screening of gene of acylglucosamine 2-epimerase

Screening of recombinant containing DNA coding for acylglucosamine 2-epimerase was carried out according to immunostaining method using antibody of example 1 and picoBlue Immunoscreening Kit (STRATAGENE).

64 positive phages were obtained by screening 1,200,000 of plaques according to said method. Optionally 12 strains were selected from said positive phages. *E. coli* was infected by said strains with f1 helper phage so as to integrate cDNAs into plasmids. *E. coli* strain with plasmid having acylglucosamine 2-epimerase activities was selected. The plasmid was taken out from *E. coli* selected to prepare a recombinant plasmid pEPI1 (4.3 kbp) containing insertional fragment (1.4 kbp). Restriction map of pEPI1 is shown in Fig. 2.

The *E. coli* into which pEPI1 (4.3 kbp) was introduced was deposited under accession number FERM BP-4602 in National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology on March 11, 1994.

(4) Determination of acylglucosamine 2-epimerase activities

In order to determine acylglucosamine 2-epimerase activities in *E. coli*, *E. coli* XL1-Blue was inoculated in LB medium (1% peptone, 0.5% yeast extract, 1% NaCl, pH 7.0) containing 100mg/liter of ampicillin and then shake-cultured. Further, for efficient production of said enzyme, isopropyl- β -D-thiogalactopyranoside was added to the medium within a final concentration of 1 mM when starting culture. The resulting medium was cultured at 37 °C for 12 hours and then centrifuged (5,000 rpm, 10 minutes) to obtain pellet of cells. Cell extract was obtained by ultrasonic disruption of cells. The cell extract was reacted at 37 °C for 30 minutes at a final volume of 0.5 ml in the presence of 40 mM of N-acetylmannosamine (NACALAI TESQUE) or 40 mM of N-acetylglucosamine (NACALAI TESQUE), 4mM of adenosinetriphosphate

(KOJIN Co., Ltd.), 10 mM of magnesium chloride and 100 mM of Tris-HCl buffer (pH 7.5). The resulting reaction mixture was boiled for 3 minutes in boiled water to stop the reaction. The enzymatic activities were determined by assaying N-acetylmannosamine or N-acetylglucosamine of reaction supernatant prepared by centrifugation (12,000 rpm, 5 minutes) of said reaction mixture. In the determination, it is difficult to determine N-acetylmannosamine and N-acetylglucosamine as they are by HPLC. In order to convert these compounds to 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives according to the method of Honda et al (Analytical Biochemistry, **180**, 351-357 (1989)), 10 µl of said reaction supernatant was removed to 1.5 ml volume of microtube, to which 50 µl of 0.5M PMP methanol solution and 50 µl of 0.3M NaOH aqueous solution was added, subsequently the mixture was reacted at 70 °C for 30 minutes. The reaction mixture was cooled at room temperature for 10 minutes, and then neutralized with 150 µl of 0.1M hydrochloric acid aqueous solution. To the neutralized solution was added 200 µl of chloroform, and the mixture was mixed to separate unreacted PMP in chloroform layer to obtain PMP-N-acetylhexosamine in aqueous layer. After removing chloroform layer, the aqueous layer was dried in vacuo. The dried residue was dissolved in 250 µl of distilled water, 10 µl of which was injected to HPLC for separation and determination. HPLC analysis was carried out using LC-6A system (SHIMADZU CORP.) and column (Cosmosil 5C18-AR (inner diameter 6.0 mm x length 150 mm), NACALAI TESQUE) with a mixture of acetonitrile and 50 mM phosphate buffer (pH 7.0) (2:8) as mobile phase at a rate of 1ml/min. Detection were performed by absorption amount of ultraviolet at 245 nm. The enzymatic activities are shown as unit. One unit is defined as activity to produce 1 µmol of N-acetylglucosamine per 1 minute from the reaction of N-acetylmannosamine as substrate.

Consequently, when N-acetylmannosamine was contained in the reaction mixture, N-acetylmannosamine was converted to N-acetylglucosamine in the presence of cell extract (see Fig.3). Similarly, N-acetylglucosamine was converted to N-acetylmannosamine in the presence of cell extract. In each reaction, N-acetylglucosamine : N-acetylmannosamine reached a equilibrium condition of 75 : 25. No conversion was observed without addition of said cell extract to said mixture. Furthermore, antibodies directed to purified acylglucosamine 2-epimerase was reacted with a band of 45,000 daltons in western blotting. The test results, as a whole, demonstrate that cloned gene produce protein corresponding to acylglucosamine 2-epimerase. Further, acylglucosamine 2-epimerase activities of the cell extract correspond to 10 unit production per 1 liter of culture medium, showing that specific activities of cell extract was 0.03U/mg, which is similar to extract of porcine kidney cortex. The results demonstrate that acylglucosamine 2-epimerase, conventionally obtained only from animal tissues, may be produced by microorganisms transformed by plasmid pEP11.

(5) Determination of nucleotide sequence of acylglucosamine 2-epimerase

A nucleotide sequence of 1.4 kbp of DNA fragment was determined by dideoxy method whose basic principle was a process described by Sanger et al (Proceedings of The National Academy of Sciences of the United States of America, **74**, 5463-5467 (1977)). Because a vector employed in pEP11 was pBluescript capable of becoming single strand, deletion mutant of the vector was prepared, and then nucleotide sequence thereof was determined. The nucleotide sequence coding for acylglucosamine 2-epimerase is shown in Fig.1. Further, amino acid sequence of polypeptide obtained by translation of said nucleotide sequence coding for the enzyme is shown simultaneously.

Example 3

Production of acylglucosamine 2-epimerase by microorganisms

(1) Construction of plasmid producing acylglucosamine 2-epimerase with high efficiency

Highly efficient production of acylglucosamine 2-epimerase by microorganisms become possible by construction of deletion plasmid of pEP11. The plasmid pEP11 (20 µg) in 500 µl solution was cut at 37 °C for 4 hours with restriction enzymes of 100 units of SacI and 100 units of XbaI. The mixture treated by restriction enzymes was treated at 75 °C for 15 minutes, extracted by phenol/chloroform (1:1) and then precipitated with ethanol. The precipitate was dissolved in sterilized water at a concentration of 1µg/µl. Tens of deletion plasmids were prepared from the solution using ExoIII/Mung Deletion Kit (STRATAGENE), in which pEP114 produced acylglucosamine 2-epimerase with high efficiency. *E. coli* XL1-Blue transformed by plasmid pEP114 was cultured in LB medium containing 100 µl/ml of ampicillin and 1mM of isopropyl-β-D-thiogalactopyranoside at 37 °C for 12 hours. Cell extract was prepared from cells of said culture. Acylglucosamine 2-epimerase activities of cell extract correspond to about 1,000 units per 1 liter of culture medium, and specific activities thereof was 1.6U/mg, which was 53 times as much as extract of porcine kidney cortex.

(2) Production of acylglucosamine 2-epimerase

The plasmid pEP114 provides useful means for producing a larger amount of acylglucosamine 2-epimerase in *E. coli*. Culture of *E. coli* to obtain acylglucosamine 2-epimerase is much easier than preparation thereof from animal tissue.

Culture thereof was carried out by inoculating *E. coli* XL1-Blue transformed by plasmid pEP114 in two shaking flasks (2-liter volume) containing 500 ml of LB medium (1% peptone, 0.5% yeast extract, 1% NaCl, pH 7.0) containing 100mg/liter of ampicillin and 1mM IPTG, then shake-cultured the flasks. After culture at 37 °C for 12 hours, cultured materials were collected by centrifugation and washed with saline two times. The cells were then suspended in 50 ml of phosphate buffer (pH 7.6) containing 1mM of EDTA and 0.05% of 2-mercaptoethanol, and then disrupted by ultrasonic oscillation (UR-200P, TOMY SEIKO CO.). Precipitate was removed by centrifugation to obtain cell extract. To the cell extract was added protamine sulfate at a concentration of 0.03%(W/V). Centrifugation was conducted as nucleic acid removal treatment. The supernatant was subjected to salting out treatment with ammonium sulfate. Fractions saturated with 20-80 % ammonium sulfate were collected and dialyzed against phosphate buffer. The dialyzed solution was applied to DEAE-cellulose (WHATMAN) column (diameter 50mm x length 100 mm) equilibrated with said phosphate buffer for adsorption thereof. Elution of adsorbed proteins was carried out by adding suitable concentration of potassium chloride to said phosphate buffer. Acylglucosamine 2-epimerase activities were existed in fractions with potassium chloride concentration of 75-100 mM, which were collected, concentrated, desalted and then applied to Q-Sepharose (PHARMACIA) (inner diameter 1cm x length 5cm). Elution of adsorbed proteins was carried out by linear gradient using said phosphate buffer containing 100 mM of potassium chloride and said phosphate buffer containing 300 mM of potassium chloride. Fractions with acylglucosamine 2-epimerase activities were collected, concentrated by said membrane filter and desalted to obtain about 700 unit (33mg) of purified cloned acylglucosamine 2-epimerase.

Example 4

Purification of acylglucosamine 2-epimerase from rat kidney

Acylglucosamine 2-epimerase from rat kidney was purified in the same manner as Example 1(1) using 300 g of rat kidney to give 0.15 mg of acylglucosamine 2-epimerase, provided that acylglucosamine 2-epimerase was purified using Mono Q column in place of reverse phase column (μ Bondasphere 5 μ C4-300 Å) which causes inactivation of enzyme.

Example 5

Inhibition of renin activities by acylglucosamine 2-epimerase (1)

A 2.5 mU of commercially available renin (SIGMA) and 0.25 pmol of cloned acylglucosamine 2-epimerase obtained in example 3 were added to 40 μ l of buffer A (0.1M sodium phosphate buffer (pH 6.5), 1mM EDTA, 1 μ M leupeptin, 0.05% bovine serum albumin). The mixture was reacted at 37 °C for 1 hour. After the reaction, 960 μ l of chilled buffer A was added thereto to adjust the total volume thereof to 1 ml. To 250 μ l of buffer A were added a part of said diluted solution thus prepared (25 μ l) and 0.4 mg/ml of angiotensinogen (SIGMA) and phenylmethylsulfonyl fluoride at a final concentration of 2.5 mM. The reaction mixture was then reacted at 37 °C for 30 minutes. The solution was treated for 3 minutes in boiled water to stop the reaction. After centrifugation (14,000 rpm, 10 minutes), angiotensin I released in supernatant was determined. The results were shown in Table 1.

Table 1

Cloned epimerase (pmol)	Remaining renin activities (%)
0	100
1	87
3	66
10	54
25	48

Example 6

Inhibition of renin activities by acylglucosamine 2-epimerase (1)

Renin inhibition activities of acylglucosamine 2-epimerase were determined in the same manner as example 5 except that rat acylglucosamine 2-epimerase obtained in example 4 was employed in place of cloned acylglucosamine

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2-epimerase obtained in example 3. The results are shown in Table 2.

Table 2

Rat kidney epimerase (pmol)	Remaining renin activities (%)
0	100
1	98
10	88
50	48

Example 7

Binding reaction of renin and acylglucosamine 2-epimerase (1)

To 100 μ l buffer A was added 25mU of renin (SIGMA) and 140 pmol of cloned acylglucosamine 2-epimerase obtained in example 3, and the resulting mixture was reacted at 37 °C for 1 hour.

Said reaction solution (100 μ l) was fractionated by gel filtration chromatography (Column, Superose 12HR 10/30; Mobile phase, 50mM sodium phosphate buffer (pH 7.5) -150mM sodium chloride; flow rate 1ml/min; Detection, ultraviolet (280 nm)). Renin activities of each fraction were assayed to determine change of molecular weight of renin.

The renin activities of fractions eluted by said gel filtration chromatography were determined according to the method of example 5. Consequently, in case of no addition of acylglucosamine 2-epimerase, renin activities were eluted at a position of about 40,000 daltons corresponding to molecular weight of renin. However, in case of addition of acylglucosamine 2-epimerase, renin activities were eluted at a position of about 60,000 daltons, demonstrating that said enzyme is attached to renin to increase molecular weight thereof.

The high-molecular-weight renin obtained in this example coincided very closely with molecular weight (about 60,000 daltons) of high-molecular-weight type (HMW) renin determined by gel filtration chromatography, i.e., the complex of renin with renin binding protein reported by Takahashi et al (J. Biochem., 93, 1583-1594 (1983)). Therefore, said acylglucosamine 2-epimerase has renin binding activities.

Example 8

Binding reaction of renin and acylglucosamine 2-epimerase (2)

Determination of molecular weight thereof was carried out by gel filtration chromatography in the same manner as example 7 except that rat acylglucosamine 2-epimerase obtained in example 4 was employed in place of cloned acylglucosamine 2-epimerase obtained in example 3. Consequently, molecular weight of renin was increased as high as 60,000 daltons as shown in example 7.

Example 9

A method for producing N-acetylmannosamine with protein having renin binding activities

Production of N-acetylmannosamine was carried out from inexpensive N-acetylglucosamine. A mixture (100ml) consisting of 10 g of N-acetylglucosamine, 5 mg of protein having renin binding activities obtained in example 3, 4 mM ATP and 10mM MgCl₂ was adjusted at a pH of 7.5, and the mixture was reacted at 37 °C for 24 hours. The reaction solution was concentrated to give syrup in vacuo. To the syrup obtained was added 40 ml of ethanol and the solution was heated in a boiled water bath for 10 minutes and then allowed to stand for 3 hours. Since N-acetylglucosamine is insoluble and N-acetylmannosamine is insoluble, precipitate (N-acetylglucosamine) was filtered off and the filtrate was concentrated to give 2 g of N-acetylmannosamine with a purity of at least 91 % in vacuo.

Example 10

Production of sialic acid using protein having renin binding activities

5 Production of N-acetylneuraminic acid was carried out by acting the protein having renin binding activities and N-acetylneuraminic acid lyase on N-acetylglucosamine and pyruvic acid.

A solution prepared by dissolving 22 g of N-acetylglucosamine and 11 g of pyruvic acid in 50 mM of Tris-HCl buffer (pH 7.5) containing 5 mM ATP and 5 mM MgCl₂. To the solution was added 15 mg of protein having renin binding activities obtained in example 3 and 500 unit of N-acetylneuraminic acid lyase, and the total volume of the solution was adjusted to 0.5 litre. The mixture was reacted at 30 °C for 48 hours. After the reaction, 12.4g of N-acetylneuraminic acid was produced in the reaction mixture. Reaction product was isolated by ion-exchange chromatography with Dowex 11 (DOW CHEMICAL CO.). After concentration, 10.3 g of N-acetylneuraminic acid was obtained by lyophilization.

Claims

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1. An acylglucosamine 2-epimerase.

2. An acylglucosamine 2-epimerase as defined in (1) to (3) below :

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(1) an acylglucosamine 2-epimerase comprising as an essential sequence said amino acid sequence represented by the formula (A);

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(2) an acylglucosamine 2-epimerase represented by the formula (A) in which at least one of positions selected from the group consisting of 10, 13, 21, 23, 27, 33, 45, 47, 51, 71, 72, 76-79, 93, 94, 101, 110, 120, 136, 137, 139, 141, 142, 145, 149, 155, 159, 162, 163, 171, 173, 174, 176, 178, 187, 195, 199-202, 205, 208, 212, 224, 232, 234, 237, 243, 249, 258-261, 263, 266, 267, 269, 270, 272, 275, 282, 287-289, 300, 301, 309, 317, 318, 328, 329, 334, 337, 348, 363, 364, 371, 392, 393, 395, 399, 401 and 402 is replaced or deleted by another amino acid; or

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(3) an acylglucosamine 2-epimerase formed by adding Pro Ala Pro Ser Pro Ala Pro Thr Pro Ala Cys Arg Gly Ala Glu; Pro Ala Pro Leu Gly Ser Leu Pro Ala Val Pro Thr Arg Glu Gly Ser Lys; or Lys Gly Asn Lys Ser Trp Gln Asp to N-terminal or C-terminal of polypeptide of formula (A); (provided that said polypeptide, in which a part of formula (A) is replaced or deleted, has acylglucosamine 2-epimerase activities.).

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(A)

5 Met Glu Lys Glu Arg Glu Thr Leu Gln Ala Trp Lys
Glu Arg Val Gly Gln Glu Leu Asp Arg Val Met Ala
10 Phe Trp Leu Glu His Ser His Asp Arg Glu His Gly
Gly Phe Phe Thr Cys Leu Gly Arg Asp Gly Arg Val
15 Tyr Asp Asp Leu Lys Tyr Val Trp Leu Gln Gly Arg
Gln Val Trp Met Tyr Cys Arg Leu Tyr Arg Lys Leu
20 Glu Arg Phe His Arg Pro Glu Leu Leu Asp Ala Ala
Lys Ala Gly Gly Glu Phe Leu Leu Arg His Ala Arg
25 Val Ala Pro Pro Glu Lys Lys Cys Ala Phe Val Leu
Thr Arg Asp Gly Arg Pro Val Lys Val Gln Arg Ser
30 Ile Phe Ser Glu Cys Phe Tyr Thr Met Ala Met Asn
Glu Leu Trp Arg Val Thr Ala Glu Ala Arg Tyr Gln
35 Ser Glu Ala Val Asp Met Met Asp Gln Ile Val His
Trp Val Arg Glu Asp Pro Ser Gly Leu Gly Arg Pro
40 Gln Leu Pro Gly Ala Val Ala Ser Glu Ser Met Ala
Val Pro Met Met Leu Leu Cys Leu Val Glu Gln Leu
45 Gly Glu Glu Asp Glu Glu Leu Ala Gly Arg Tyr Ala
Gln Leu Gly His Trp Cys Ala Arg Arg Ile Leu Gln
50 His Val Gln Arg Asp Gly Gln Ala Val Leu Glu Asn

55

5 Val Ser Glu Asp Gly Glu Glu Leu Ser Gly Cys Leu
 Gly Arg His Gln Asn Pro Gly His Ala Leu Glu Ala
 10 Gly Trp Phe Leu Leu Arg His Ser Ser Arg Ser Gly
 Asp Ala Lys Leu Arg Ala His Val Ile Asp Thr Phe
 Leu Leu Leu Pro Phe Arg Ser Gly Trp Asp Ala Asp
 15 His Gly Gly Leu Phe Tyr Phe Gln Asp Ala Asp Gly
 Leu Cys Pro Thr Gln Leu Glu Trp Ala Met Lys Leu
 20 Trp Trp Pro His Ser Glu Ala Met Ile Ala Phe Leu
 Met Gly Tyr Ser Glu Ser Gly Asp Pro Ala Leu Leu
 25 Arg Leu Phe Tyr Gln Val Ala Glu Tyr Thr Phe Arg
 Gln Phe Arg Asp Pro Glu Tyr Gly Glu Trp Phe Gly
 30 Tyr Leu Asn Arg Glu Gly Lys Val Ala Leu Thr Ile
 Lys Gly Gly Pro Phe Lys Gly Cys Phe His Val Pro
 35 Arg Cys Leu Ala Met Cys Glu Glu Met Leu Ser Ala
 Leu Leu Ser Arg Leu Ala

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3. A DNA molecule coding for acylglucosamine 2-epimerase.

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4. A DNA molecule according to claim 3 comprising a nucleotide sequence coding for amino acid sequence of the formula (A) or a nucleotide sequence coding for an amino acid sequence of formula (A) a part of which is replaced or deleted (provided that said amino acid sequence, a part of which is replaced or deleted, codes for polypeptide having acylglucosamine 2-epimerase activities.).

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5. A DNA molecule according to claim 4 comprising a nucleotide sequence of the following formula (X):

(X)

5 ATG GAG AAG GAG CGC GAA ACT CTG CAG GCC TGG AAG
GAG CGT GTG GGC CAA GAG CTG GAC CGC GTG ATG GCT
10 TTC TGG CTG GAG CAC TCC CAC GAT CGG GAG CAC GGG
GGC TTC TTC ACG TGC CTG GGC CGC GAC GGG CGG GTG
15 TAT GAC GAC CTC AAG TAC GTC TGG CTG CAG GGG AGG
CAG GTG TGG ATG TAC TGT CGC CTG TAC CGC AAG CTT
20 GAG CGC TTC CAC CGC CCT GAG CTT CTG GAT GCG GCT
AAA GCA GGG GGC GAA TTT TTG CTG CGC CAT GCC CGA
25 GTG GCA CCT CCT GAA AAG AAG TGT GCC TTT GTG CTG
ACG CGG GAC GGC CGG CCC GTC AAG GTG CAG CGG AGC
30 ATC TTC AGT GAG TGC TTC TAC ACC ATG GCC ATG AAC
GAG CTG TGG AGG GTG ACG GCG GAG GCA CGG TAC CAG
35 AGC GAA GCG GTG GAC ATG ATG GAT CAG ATC GTG CAC
TGG GTG CGA GAG GAC CCC TCT GGG CTG GGC CGG CCC
40 CAG CTC CCC GGG GCC GTG GCC TCG GAG TCC ATG GCA
GTG CCC ATG ATG CTG CTG TGC CTG GTG GAG CAG CTC
45 GGG GAG GAG GAC GAG GAG CTG GCA GGC CGC TAC GCG
CAG CTG GGG CAC TGG TGC GCT CGG AGG ATC CTG CAG
50 CAC GTC CAG AGG GAT GGA CAG GCT GTG CTG GAG AAT

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5 GTG TCG GAA GAT GGC GAG GAA CTT TCT GGC TGC CTG
 GGG AGA CAC CAG AAC CCA GGC CAC GCG CTG GAA GCT
 10 GGC TGG TTC CTG CTC CGC CAC AGC AGC CGG AGC GGT
 GAC GCC AAA CTT CGA GCC CAC GTC ATC GAC ACG TTC
 CTG CTA CTG CCT TTC CGC TCC GGA TGG GAC GCT GAT
 15 CAC GGA GGC CTC TTC TAC TTC CAG GAT GCC GAT GGC
 CTC TGC CCC ACC CAG CTG GAG TGG GCC ATG AAG CTC
 20 TGG TGG CCG CAC AGC GAA GCC ATG ATC GCC TTT CTC
 ATG GGC TAC AGT GAG AGC GGG GAC CCT GCC TTA CTG
 25 CGT CTC TTC TAC CAG GTG GCC GAG TAC ACG TTT CGC
 CAG TTT CGT GAT CCC GAG TAC GGG GAA TGG TTT GGC
 30 TAC CTG AAC CGA GAG GGG AAG GTT GCC CTC ACT ATC
 AAG GGG GGT CCC TTT AAA GGC TGC TTC CAC GTG CCG
 35 CGG TGC CTT GCC ATG TGC GAA GAG ATG CTG AGC GCC
 CTG CTG AGC CGC CTC GCC TAG

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6. A recombinant vector into which a DNA molecule coding for acylglucosamine 2-epimerase is integrated.
7. A recombinant vector according to claim 6 comprising a nucleotide sequence coding for the amino acid sequence of the formula (A) or a nucleotide sequence coding for an amino acid sequence of formula (A) a part of which is replaced or deleted (provided that said amino acid sequence, a part of which is replaced or deleted, codes for polypeptide having acylglucosamine 2-epimerase activities.).
8. A recombinant vector according to claim 7 comprising a nucleotide sequence coding for the amino acid sequence of the formula (X).
9. A transformant wherein a recombinant vector comprising the DNA molecule of claim 3 is introduced into said transformant.
10. A method for producing acylglucosamine 2-epimerase comprising introducing a recombinant vector into which a DNA molecule coding for acylglucosamine 2-epimerase is integrated into a cell to form a transformant, culturing

said transformant in medium to produce and accumulate acylglucosamine 2-epimerase in culture, and collecting acylglucosamine 2-epimerase from the culture.

11. An acylglucosamine 2-epimerase having renin binding activities.

12. An epimerase according to claim 11 comprising as an essential component a polypeptide as defined in (1) to (3) below:

(1) a polypeptide comprising as an essential sequence said amino acid sequence represented by the formula (A);

(2) a polypeptide represented by the formula (A) in which at least one of positions selected from the group consisting of 10, 13, 21, 23, 27, 33, 45, 47, 51, 71, 72, 76-79, 93, 94, 101, 110, 120, 136, 137, 139, 141, 142, 145, 149, 155, 159, 162, 163, 171, 173, 174, 176, 178, 187, 195, 199-202, 205, 208, 212, 224, 232, 234, 237, 243, 249, 258-261, 263, 266, 267, 269, 270, 272, 275, 282, 287-289, 300, 301, 309, 317, 318, 328, 329, 334, 337, 348, 363, 364, 371, 392, 393, 395, 399, 401 and 402 is replaced or deleted by another amino acid; or

(3) a polypeptide formed by adding Pro Ala Pro Ser Pro Ala Pro Thr Pro Ala Cys Arg Gly Ala Glu; Pro Ala Pro Leu Gly Ser Leu Pro Ala Val Pro Thr Arg Glu Gly Ser Lys; or Lys Gly Asn Lys Ser Trp Gln Asp to N-terminal or C-terminal of polypeptide of formula (A); (provided that said polypeptide, in which a part of formula (A) is replaced or deleted, has acylglucosamine 2-epimerase activities.).

13. A polypeptide of any of (1) to (3) below (provided that polypeptides represented by formula (R-1), (R-2) and (R-3) is excluded.);

(1) a polypeptide comprising as an essential sequence said amino acid sequence represented by the formula (A);

(2) a polypeptide represented by the formula (A) in which at least one of positions selected from the group consisting of 10, 13, 21, 23, 27, 33, 45, 47, 51, 71, 72, 76-79, 93, 94, 101, 110, 120, 136, 137, 139, 141, 142, 145, 149, 155, 159, 162, 163, 171, 173, 174, 176, 178, 187, 195, 199-202, 205, 208, 212, 224, 232, 234, 237, 243, 249, 258-261, 263, 266, 267, 269, 270, 272, 275, 282, 287-289, 300, 301, 309, 317, 318, 328, 329, 334, 337, 348, 363, 364, 371, 392, 393, 395, 399, 401 and 402 is replaced or deleted by another amino acid; or

(3) a polypeptide formed by adding Pro Ala Pro Ser Pro Ala Pro Thr Pro Ala Cys Arg Gly Ala Glu; Pro Ala Pro Leu Gly Ser Leu Pro Ala Val Pro Thr Arg Glu Gly Ser Lys; or Lys Gly Asn Lys Ser Trp Gln Asp to N-terminal or C-terminal of polypeptide of formula (A);

(R-1)

5 Met Glu Lys Glu Arg Glu Thr Leu Gln Ala Trp Lys
Glu Arg Val Gly Gln Glu Leu Asp Arg Val Val Ala
10 Phe Trp Met Glu His Ser His Asp Gln Glu His Gly
Gly Phe Phe Thr Cys Leu Gly Arg Glu Gly Arg Val
15 Tyr Asp Asp Leu Lys Tyr Val Trp Leu Gln Gly Arg
Gln Val Trp Met Tyr Cys Arg Leu Tyr Arg Thr Phe
20 Glu Arg Phe Arg His Ala Gln Leu Leu Asp Ala Ala
Lys Ala Gly Gly Glu Phe Leu Leu Arg Tyr Ala Arg
25 Val Ala Pro Pro Gly Lys Lys Cys Ala Phe Val Leu
Thr Arg Asp Gly Arg Pro Val Lys Val Gln Arg Thr
30 Ile Phe Ser Glu Cys Phe Tyr Thr Met Ala Met Asn
Glu Leu Trp Arg Ala Thr Gly Glu Val Arg Tyr Gln
35 Thr Glu Ala Val Glu Met Met Asp Gln Ile Val His
Trp Val Gln Glu Asp Ala Ser Gly Leu Gly Arg Pro
40 Gln Leu Gln Gly Ala Pro Ala Ala Glu Pro Met Ala
Val Pro Met Met Leu Leu Asn Leu Val Glu Gln Leu
45 Gly Glu Ala Asp Glu Glu Leu Ala Gly Lys Tyr Ala
Glu Leu Gly Asp Trp Cys Ala Arg Arg Ile Leu Gln

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His Val Gln Arg Asp Gly Gln Ala Val Leu Glu Asn
5 Val Ser Glu Gly Gly Lys Glu Leu Pro Gly Cys Leu
Gly Arg Gln Gln Asn Pro Gly His Thr Leu Glu Ala
10 Gly Trp Phe Leu Leu Arg His Cys Ile Arg Lys Gly
Asp Pro Glu Leu Arg Ala His Val Ile Asp Lys Phe
15 Leu Leu Leu Pro Phe His Ser Gly Trp Asp Pro Asp
His Gly Gly Leu Phe Tyr Phe Gln Asp Ala Asp Asn
20 Phe Cys Pro Thr Gln Leu Glu Trp Ala Met Lys Leu
Trp Trp Pro His Ser Glu Ala Met Ile Ala Phe Leu
25 Met Gly Tyr Ser Asp Ser Gly Asp Pro Val Leu Leu
Arg Leu Phe Tyr Gln Val Ala Glu Tyr Thr Phe Arg
30 Gln Phe Arg Asp Pro Glu Tyr Gly Glu Trp Phe Gly
Tyr Leu Ser Arg Glu Gly Lys Val Ala Leu Ser Ile
35 Lys Gly Gly Pro Phe Lys Gly Cys Phe His Val Pro
Arg Cys Leu Ala Met Cys Glu Glu Met Leu Gly Ala
40 Leu Leu Ser Arg Pro Ala Pro Ala Pro Ser Pro Ala
Pro Thr Pro Ala Cys Arg Gly Ala Glu

(R-2)

5 Met Glu Lys Glu Arg Glu Thr Leu Gln Val Trp Lys
Gln Arg Val Gly Gln Glu Leu Asp Ser Val Ile Ala
10 Phe Trp Met Glu His Ser His Asp Gln Glu His Gly
Gly Phe Phe Thr Cys Leu Gly Arg Asp Gly Gln Val
15 Tyr Asp His Leu Lys Tyr Val Trp Leu Gln Gly Arg
Gln Val Trp Met Tyr Cys Arg Leu Tyr Arg Thr Phe
20 Glu Arg Phe Arg Arg Val Glu Leu Leu Asp Ala Ala
Lys Ala Gly Gly Glu Phe Leu Leu Ser Tyr Ala Arg
25 Val Ala Pro Pro Gly Lys Lys Cys Ala Phe Val Leu
Thr Gln Asp Gly Arg Pro Val Lys Val Gln Arg Thr
30 Ile Phe Ser Glu Cys Phe Tyr Thr Met Ala Met Asn
Glu Leu Trp Lys Val Thr Gly Glu Met His Tyr Gln
35 Arg Glu Ala Val Glu Met Met Asp Gln Ile Ile His
Trp Val Arg Glu Asp Pro Ala Gly Leu Gly Arg Pro
40 Gln Leu Ser Gly Thr Leu Ala Thr Glu Pro Met Ala
Val Pro Met Met Leu Leu Asn Leu Val Glu Gln Leu
45 Gly Glu Glu Asp Glu Glu Met Thr Asp Lys Tyr Ala
Glu Leu Gly Asp Trp Cys Ala His Arg Ile Leu Gln

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His Val Gln Arg Asp Gly Gln Val Val Leu Glu Asn
5 Val Ser Glu Asp Gly Lys Glu Leu Pro Gly Cys Leu
Gly Arg His Gln Asn Pro Gly His Thr Leu Glu Ala
10 Gly Trp Phe Leu Leu Gln Tyr Ala Leu Arg Lys Gly
Asp Pro Lys Leu Gln Arg His Ile Ile Asp Lys Phe
15 Leu Leu Leu Pro Phe His Ser Gly Trp Asp Pro Glu
His Gly Gly Leu Phe Tyr Phe Gln Asp Ala Asp Asp
20 Leu Cys Pro Thr Gln Leu Glu Trp Asn Met Lys Leu
Trp Trp Pro His Thr Glu Ala Met Ile Ala Phe Leu
25 Met Gly Tyr Arg Asp Ser Gly Asp Pro Ala Leu Leu
Asn Leu Phe Tyr Gln Val Ala Glu Tyr Thr Phe His
30 Gln Phe Arg Asp Pro Glu Tyr Gly Glu Trp Phe Gly
Tyr Leu Asn Gln Glu Gly Lys Val Ala Leu Thr Ile
35 Lys Gly Gly Pro Phe Lys Gly Cys Phe His Val Pro
Arg Cys Leu Ala Met Cys Glu Gln Ile Leu Gly Ala
40 Leu Leu Gln Arg Leu Gly Pro Ala Pro Leu Gly Ser
Leu Pro Ala Val Pro Thr Arg Glu Gly Ser Lys

Met Glu Lys Glu Arg Glu Thr Leu Gln Ala Trp Lys
 5 Glu Arg Val Gly Gln Glu Leu Asp Arg Val Met Ala
 Phe Trp Leu Glu His Ser His Asp Arg Glu His Gly
 10 Gly Phe Phe Thr Cys Leu Gly Arg Asp Gly Arg Val
 Tyr Asp Asp Leu Lys Tyr Val Trp Leu Gln Gly Arg
 15 Gln Val Trp Met Tyr Cys Arg Leu Tyr Arg Lys Leu
 Glu Arg Phe His Arg Pro Glu Leu Leu Asp Ala Ala
 20 Lys Ala Gly Gly Glu Phe Leu Leu Arg His Ala Arg
 Val Ala Pro Pro Glu Lys Lys Cys Ala Phe Val Leu
 25 Thr Arg Asp Gly Arg Pro Val Lys Val Gln Arg Ser
 Ile Phe Ser Glu Cys Phe Tyr Thr Met Ala Met Asn
 30 Glu Leu Trp Arg Val Thr Ala Glu Ala Arg Tyr Gln
 Ser Glu Ala Val Glu Met Met Asp Gln Ile Val His
 35 Trp Val Arg Glu Asp Pro Ser Gly Leu Gly Arg Pro
 Gln Leu Pro Gly Ala Val Ala Ser Glu Ser Met Ala
 40 Val Pro Met Met Leu Leu Cys Leu Val Glu Gln Leu
 Gly Glu Glu Asp Glu Glu Leu Ala Gly Arg Tyr Ala
 45 Gln Leu Gly His Trp Cys Ala Arg Arg Ile Leu Gln
 His Val Gln Arg Asp Gly Gln Ala Val Leu Glu Asn

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Val Ser Glu Asp Gly Glu Glu Leu Ser Gly Cys Leu
 Gly Arg His Gln Asn Pro Gly His Ala Leu Glu Ala
 Gly Trp Phe Leu Leu Arg His Ser Ser Arg Ser Gly
 Asp Ala Lys Leu Arg Ala His Val Ile Asp Thr Phe
 Leu Leu Leu Pro Phe Arg Ser Gly Trp Asp Ala Asp
 Tyr Gly Gly Leu Phe Tyr Phe Gln Asp Ala Asp Gly
 Leu Cys Pro Thr Gln Leu Glu Trp Ala Met Lys Leu
 Trp Trp Pro His Arg Gln Ala Met Ile Ala Phe Leu
 Met Gly Tyr Ser Glu Ser Gly Asp Pro Ala Leu Leu
 Arg Leu Phe Tyr Gln Val Ala Glu Tyr Thr Phe Arg
 Gln Phe Arg Asp Pro Glu Tyr Gly Glu Trp Phe Gly
 Tyr Leu Asn Arg Glu Gly Lys Val Ala Leu Thr Ile
 Lys Gly Gly Pro Phe Lys Gly Cys Phe His Val Pro
 Arg Cys Leu Ala Met Cys Glu Glu Met Leu Ser Ala
 Leu Leu Ser Arg Leu Ala

14. A DNA molecule coding for polypeptide according to claim 13.

15. A DNA molecule according to claim 14 comprising the nucleotide sequence of formula (X).

16. A recombinant vector into which a DNA molecule coding for acylglucosamine 2-epimerase according to claim 14 or 15 is integrated.

17. A transformant wherein a recombinant vector according to claim 16 is introduced into said transformant.

18. A method for producing acylglucosamine 2-epimerase having renin binding activities comprising introducing a recombinant vector into which a DNA molecule coding for acylglucosamine 2-epimerase having renin binding activities is integrated into a cell to form a transformant, culturing said transformant in medium to produce and accumulate acylglucosamine 2-epimerase in culture, and collecting acylglucosamine 2-epimerase from the culture.

19. An antihypertensive agent comprising as an essential component acylglucosamine 2-epimerase or a derivative thereof according to claim 11 or 12.

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20. An epimerizing agent converting N-acetylglucosamine to N-acetylmannosamine comprising as an essential component a polypeptide having renin binding activities.

21. A method for producing N-acetylmannosamine comprising acting a polypeptide having renin binding activities on N-acetylglucosamine.

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22. A method for producing N-acetyneuraminic acid comprising acting a polypeptide having renin binding activities and N-acetyneuraminic acid lyase on N-acetylglucosamine and pyruvic acid.

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F I G. 1 (1)

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5'  ATG GAG AAG GAG OGC GAA ACT CTG CAG GGC TGG AAG GAG OGT GTG GGC CAA GAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Met Glu Lys Glu Arg Glu Thr Leu Gln Ala Trp Lys Glu Arg Val Gly Gln Glu

    CTG GAC OGC GTG ATG GCT TTC TGG CTG GAG CAC TOC CAC GAT CGG GAG CAC GGG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Leu Asp Arg Val Met Ala Phe Trp Leu Glu His Ser His Asp Arg Glu His Gly

    GGC TTC TTC ACG TGC CTG GGC OGC GAC GGG OGG GTG TAT GAC GAC CTC AAG TAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Gly Phe Phe Thr Cys Leu Gly Arg Asp Gly Arg Val Tyr Asp Asp Leu Lys Tyr

    GTC TGG CTG CAG GGG AGG CAG GTG TGG ATG TAC TGT OGC CTG TAC OGC AAG CTT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Val Trp Leu Gln Gly Arg Gln Val Trp Met Tyr Cys Arg Leu Tyr Arg Lys Leu

    GAG OGC TTC CAC OGC OCT GAG CTT CTG GAT GCG GCT AAA GCA GGG GGC GAA TTT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Glu Arg Phe His Arg Pro Glu Leu Leu Asp Ala Ala Lys Ala Gly Gly Glu Phe

    TTG CTG OGC CAT GCC CGA GTG GCA OCT OCT GAA AAG AAG TGT GCC TTT GTG CTG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Leu Leu Arg His Ala Arg Val Ala Pro Pro Glu Lys Lys Cys Ala Phe Val Leu

    ACG OGG GAC GGC CGG CCC GTC AAG GTG CAG OGG AGC ATC TTC AGT GAG TGC TTC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Thr Arg Asp Gly Arg Pro Val Lys Val Gln Arg Ser Ile Phe Ser Glu Cys Phe

    TAC ACC ATG GCC ATG AAC GAG CTG TGG AGG GTG ACG GCG GAG GCA CGG TAC CAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Tyr Thr Met Ala Met Asn Glu Leu Trp Arg Val Thr Ala Glu Ala Arg Tyr Gln

    AGC GAA GCG GTG GAC ATG ATG GAT CAG ATC GTG CAC TGG GTG CGA GAG GAC CCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ser Glu Ala Val Asp Met Met Asp Gln Ile Val His Trp Val Arg Glu Asp Pro

    TCT GGG CTG GGC CGG CCC CAG CTC CCC GGG GGC GTG GCC TCG GAG TOC ATG GCA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ser Gly Leu Gly Arg Pro Gln Leu Pro Gly Ala Val Ala Ser Glu Ser Met Ala

    GTG CCC ATG ATG CTG CTG TGC CTG GTG GAG CAG CTC GGG GAG GAG GAC GAG GAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Val Pro Met Met Leu Leu Cys Leu Val Glu Gln Leu Gly Glu Glu Asp Glu Glu

    CTG GCA GGC CGC TAC GCG CAG CTG GGG CAC TGG TGC GCT CGG AGG ATC CTG CAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Leu Ala Gly Arg Tyr Ala Gln Leu Gly His Trp Cys Ala Arg Arg Ile Leu Gln

    CAC GTC CAG AGG GAT GGA CAG GCT GTG CTG GAG AAT GTG TCG GAA GAT GGC GAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    His Val Gln Arg Asp Gly Gln Ala Val Leu Glu Asn Val Ser Glu Asp Gly Glu

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F I G. 1 (2)

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GAA CTT TCT GGC TGC CTG GGG AGA CAC CAG AAC OCA GGC CAC GCG CTG GAA GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Glu Leu Ser Gly Cys Leu Gly Arg His Gln Asn Pro Gly His Ala Leu Glu Ala

GGC TGG TTC CTG CTC CGC CAC AGC AGC CCG AGC GGT GAC GCC AAA CTT CGA GCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gly Trp Phe Leu Leu Arg His Ser Ser Arg Ser Gly Asp Ala Lys Leu Arg Ala

CAC GTC ATC GAC ACG TTC CTG CTA CTG OCT TTC CCG TCC GGA TGG GAC GCT GAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
His Val Ile Asp Thr Phe Leu Leu Leu Pro Phe Arg Ser Gly Trp Asp Ala Asp

CAC GGA GGC CTC TTC TAC TTC CAG GAT GGC GAT GGC CTC TGC CCC ACC CAG CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
His Gly Gly Leu Phe Tyr Phe Gln Asp Ala Asp Gly Leu Cys Pro Thr Gln Leu

GAG TGG GCC ATG AAG CTC TGG TGG CCG CAC AGC GAA GCC ATG ATC GCC TTT CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Glu Trp Ala Met Lys Leu Trp Trp Pro His Ser Glu Ala Met Ile Ala Phe Leu

ATG GGC TAC AGT GAG AGC GGG GAC CCT GCC TTA CTG CGT CTC TTC TAC CAG GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Met Gly Tyr Ser Glu Ser Gly Asp Pro Ala Leu Leu Arg Leu Phe Tyr Gln Val

GCC GAG TAC ACG TTT CGC CAG TTT CGT GAT CCC GAG TAC GGG GAA TGG TTT GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ala Glu Tyr Thr Phe Arg Gln Phe Arg Asp Pro Glu Tyr Gly Glu Trp Phe Gly

TAC CTG AAC CGA GAG GGG AAG GTT GCC CTC ACT ATC AAG GGG GGT CCC TTT AAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Tyr Leu Asn Arg Glu Gly Lys Val Ala Leu Thr Ile Lys Gly Gly Pro Phe Lys

GGC TGC TTC CAC GTG CCG CGG TGC CTT GCC ATG TGC GAA GAG ATG CTG AGC GCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gly Cys Phe His Val Pro Arg Cys Leu Ala Met Cys Glu Glu Met Leu Ser Ala

CTG CTG AGC CGC CTC GCC TAG 3'
--- --- --- --- --- ---
Leu Leu Ser Arg Leu Ala ***

```

FIG. 2

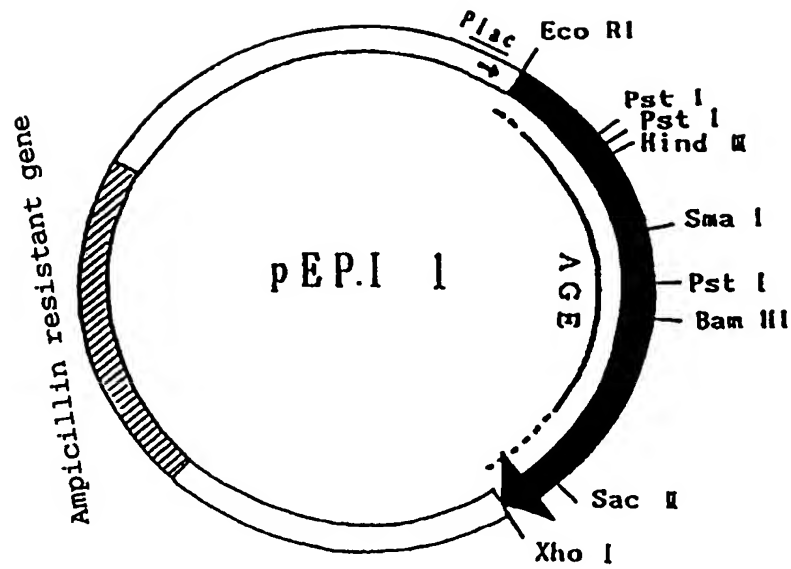
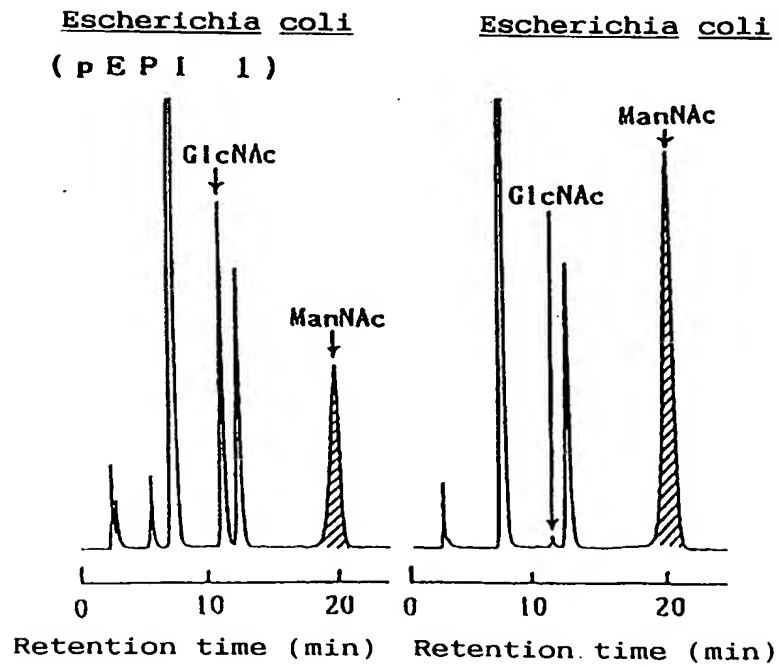
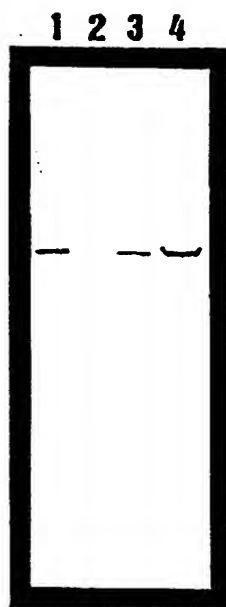


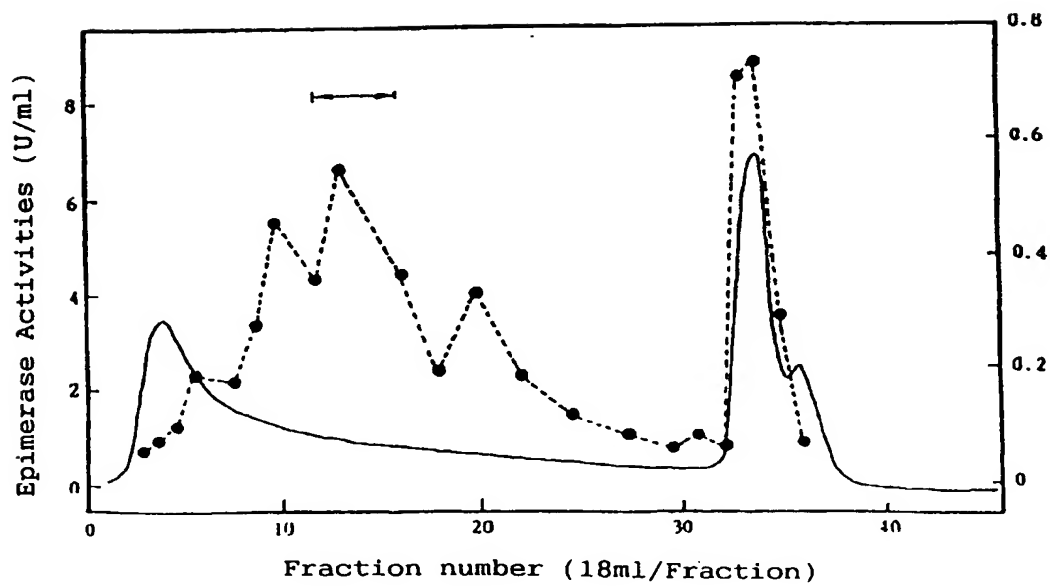
FIG. 3



F I G . 4



F I G . 5



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP95/00541

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl⁶ C12N9/90, C12N15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl⁶ C12N9/90, C12N15/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS PREVIEWS, WPI, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP, 3-180190, A (Forschungszentrum Juelich GmbH.), August 6, 1991 (06. 08. 91) & EP, 428947, A & DE, 3937891, A & AU, 9065872, A & CA, 2029984, A & US, 5071750, A & IL, 96307, A	1, 21, 22
A	Journal of Biochemistry, Vol. 110, No. 4, (1991), H. Inoue et al. "Genetic and Molecular properties of human and rat renin-binding proteins with reference to the function of the leucine zipper motif" p. 493-500	2 - 20
A	Journal of Biological Chemistry, Vol. 265, No. 12, (1990), H. Inoue et al. "Molecular cloning and sequence analysis of a cDNA encoding a porcine Ridney renin-binding protein" p. 6556-61	2 - 20

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

June 9, 1995 (09. 06. 95)

Date of mailing of the international search report

June 27, 1995 (27. 06. 95)

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